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13. ABSTRACT (Maximum 200 Words) 20 to 30% of human breast cancers amplify and overexpress ErbB2 (HER2, Neu). To examine the role of this receptor tyrosine kinase in both normally mammary gland development and in mammary tumorigenesis, I have created several mouse models. To examine the role of ErbB2 in mouse mammary development I have utilized Cre/loxP technology to create a mammary specific knockout of ErbB2. Although the loxP flanked construct was shown to be functional, as were the transgenic mice expressing Cre in the mammary epithelium, we have not yet observed mammary specific inactivation of ErbB2. This is likely due to background specific methylation of the transgene and we have completely introgressed the mice onto an FVB background. To examine the role of ErbB2 in mammary tumorigenesis, I have created a mouse model that expresses activated ErbB2 specifically in the mammary gland. This resulted in mammary adenocarcinomas that amplified and overexpressed ErbB2, closely mimicking the human condition. Further, the mammary epithelium escapes from the normal confines of the stroma in these mice. Additional experiments have revealed double minute chromosomes, overexpression of various genes and I have now isolated three cell lines from the tumors in order to examine these tumors more fully.				
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Introduction

The research objectives outlined in the initial proposal were twofold, to determine the role of ErbB2 (Neu, HER2) in normal mammary gland development and to elucidate the role of ErbB2 in mammary tumorigenesis. This work was initially based on the observation that 20-30 % of human breast cancers contain amplified copy numbers of HER2 and overexpress the proto-oncogene(1-4). Upon characterization of ErbB2 as a member of the EGFR family, a knockout was created which was embryonically lethal(5). Unfortunately, due to the early lethality, the role in mammary gland development in these mice could not be addressed. Further experiments revealed that the mammary gland expressed ErbB2 (6) but did not suggest how ErbB2 was involved in mammary gland development. While the role of ErbB2 in mammary gland development has not been elucidated, the role in tumorigenesis has been examined. Expression of the activated form of ErbB2 under the control of the Mouse Mammary Tumor Virus Promoter / Enhancer (MMTV-ErbB2) resulted in mammary tumorigenesis by 89 days in 50% of female mice (8-9). Interestingly, when the same experiment was repeated using the wild type allele, tumorigenesis was observed in 50% of female mice by 205 days(10). While defining a role for ErbB2 in mammary tumorigenesis, these results were based on expression of ErbB2 under the control of a viral promoter of questionable relevance to the human condition. Accordingly, I have sought to create mouse models where ErbB2 is specifically deleted in the mammary gland and where activated ErbB2 is expressed under the control of the endogenous promoter in the mammary gland. We have not yet observed an effect of lack of ErbB2 in mammary gland development due to loss of expression of the Cre transgene. However, I have observed tumorigenesis, amplification and overexpression of ErbB2 when the activated allele is expressed under the endogenous promoter in the mammary gland. Further analysis of these mice has revealed that the tumors contain double minute chromosomes, that the epithelium of the mammary gland can escape the normal confines of the fat pad and that the tumors are more differentiated with respect to the MMTV-ErbB2 mediated tumors.

Research Accomplishments

1. Mammary Specific Expression of Activated ErbB2 under the control of the endogenous promoter results in tumorigenesis.

One of the two major goals outlined in the initial proposal was the expression of an activated form of ErbB2 in the mammary gland under the control of the endogenous promoter. It was hoped that this would result in a better mouse model of ErbB2 mediated tumorigenesis. I have achieved the goal of expressing activated ErbB2 under the endogenous promoter specifically in the mammary gland and have observed alterations in mammary gland development. Previously I noted increased side branching and lobuloalveolar side buds in these mice prior to the onset of tumorigenesis. Interestingly, tumorigenesis in these mice required amplification and overexpression of ErbB2, mirroring the human condition (Results described in the previous annual summary and in the published manuscript (10)). However, I have noted an additional phenotype in these mice where the mammary epithelium is able to escape the normal confines of the fat pad. Further, these outgrowths usually appear to invade the stroma surrounding blood vessels in the mammary gland (Figure 1). Interestingly, this result bears a striking similarity to another mouse model created in Dr. Muller's laboratory. As part of my training under Dr.

Muller, I have created several strains of transgenic mice for use by our laboratory and by our collaborators. One of these strains, MMTV-MUC4, has the MUC4 transgene expressed tightly in the mammary gland (Figures 2 and 3) and has exhibited a very similar phenotype where the mammary epithelium escapes the confines of the normal mammary stroma (Figure 4). Interestingly, MUC4 has been described as a putative ligand for ErbB2 and the similarities in mammary phenotypes lends credence to that argument that MUC4 is indeed a ligand for ErbB2.

When compared to the MMTV-ErbB2 tumors, tumors arising from the conditional activation of ErbB2 have also exhibited another important difference. The MMTV directed tumors frequently metastasize to the lungs and the mammary glands often contain multifocal tumors. In contrast, the conditional activation of ErbB2 in the mammary gland resulted in focal tumors that very rarely metastasized to the lung. To further characterize ErbB2 mediated tumorigenesis in this model we have collaborated to employ FISH, SKY and CGH analysis and have identified double minute chromosomes and numerous karyotypic abnormalities in these tumors. The submitted manuscript that fully describes this work has been attached. Additionally, to explore the differences between the conditionally activated model and the MMTV directed model, we conducted a RNA chip experiment. The major results of this experiment are illustrated in Table 1. Briefly, this data suggests that the conditionally activated tumors are more differentiated and suggests that the ErbB2 amplified region contains other genes. After confirmation of the chip data (Figure 5), and examination of the amplified area (Figure 6), we have noted that several genes are amplified with ErbB2. Strikingly, reports of human HER2 mediated tumorigenesis have reported similar results (11-12), lending further support to the relevance of this mouse model of the human condition.

In order to examine Neu mediated tumorigenesis in this model more completely, I have now isolated three tumor cell lines that overexpress ErbB2 at high levels (Figure 7). Of the two lines tested, one has reproducibly generated tumors upon flank injections (line TM22). Interestingly, one of these tumor cell lines also overexpresses Grb7 (line TM15). These cell lines will allow the rapid identification of active signaling pathways and also will be of use to others interested in treatment of ErbB2 mediated tumorigenesis where amplification of ErbB2 has occurred.

2. Inactivation of ErbB2 in the mammary gland.

The second major goal defined in the initial proposal was to examine mammary gland development in the absence of ErbB2. To achieve this goal, I have placed loxP sites on either side of the ErbB2 cDNA and this loxP flanked cDNA, followed by a selectable marker has replaced exon 1 one of the wild type allele. The recombinant alleles ($ErbB2^{lox/lox}$) function as wild type alleles until Cre mediated recombination occurs. At that point, the recombinant alleles are excised and null alleles are created. Upon interbreeding the MMTV-Cre transgenic mice with the $ErbB2^{lox/lox}$ mice, I have observed a loss of Cre expression in the mammary gland. This initially occurred in the mixed background and subsequently occurred in a 98 % FVB background. To exclude the possibility of methylation induced transgene silencing, I have now interbred the $ErbB2^{lox/lox}$ mice 12 generations into the FVB background. I have also now interbred the MMTV-Cre transgenics with the FVB $ErbB2^{lox/lox}$ mice and am now interbreeding their heterozygous offspring. Given the absence of excision in the first two attempts to create a mammary specific knockout, we needed to ensure that the $ErbB2^{lox/lox}$ mice are functional. Accordingly, I have interbred them with transgenic mice expressing Cre under the control of the

muscle creatine kinase promoter. Importantly, this has revealed that excision is possible in the ErbB2^{flx/flx} mice and that complete excision results in a lack of ErbB2 protein (Figure 8). This experiment served to eliminate the possibility that the lack of excision observed in the MMTV-Cre ErbB2^{flx/flx} mice was due to problems with the ErbB2^{flx/flx} construct. Therefore, we are left with several possibilities for the role of ErbB2 in mammary gland development, which will be defined as the MMTV-Cre FVB ErbB2^{flx/flx} mice are analyzed. One possibility is that ErbB2 function is critical for survival of mammary epithelium and only mammary epithelium not expressing Cre recombinase which will express ErbB2 is able to survive. Interestingly, culture of ErbB2^{flx/flx} mammary epithelium and infection with an adenovirus expressing Cre recombinase did not result in mammary epithelium when reintroduced to a cleared mammary gland. However, it should be noted that this experiment has only been repeated in eight mice and is still preliminary. Additionally preliminary data is also shown (Figure 9) for culture of mammary epithelium from ErbB2^{flx/flx} mice with and without the addition of an adenovirus containing Cre recombinase. It should be noted that infection in these mammospheres occurred after the cells were seeded onto the complex basement membrane and that infection was incomplete as monitored by an additional adenovirus containing B-gal (results not shown). Thus, while there are small differences in the various culture conditions, these results are still very preliminary. What should be noted is that although the level of ErbB2 has been reduced by 90% using this construct (Chan, Hardy, Muller submitted), the mammospheres are still able to form.

3. Hormonal Regulation of ErbB2 mediated tumorigenesis.

Although not extensively described in the scope of the main report, a minor goal addressed in the original statement of work was the examination of hormonal regulation of ErbB2 mediated tumorigenesis. The conditional activation of ErbB2 by expressing the activated allele under the control of the endogenous promoter is subject to the normal hormonal control. Indeed, this is a superior model to the MMTV based systems as the MMTV promoter is subject to hormonal regulation itself. As an initial experiment, I have ovariectomized 10 mice that are expressing the activated allele under the control of the endogenous promoter as described in section 1. The onset of tumorigenesis in these mice has not differed from the non-ovariectomized controls, indicating that tumorigenesis is not dependent on hormonal signals. However, to further explore the role of hormonal regulation of ErbB2 mediated tumorigenesis, Dr. Muller has initiated a collaboration where we will examine the effect of interbreeding the conditionally activated ErbB2 mice described in section one with transgenic mice overexpressing an activated form of the Estrogen Receptor in the mammary gland. Interestingly, the amplification described in section one describes the amplification of CAB1 with ErbB2. This gene has high homology to a steroidogenic regulatory protein and may well impact hormonal regulation since ErbB2 is estrogen responsive. The amplification of CAB1 was explored in Figure Six and was shown to be co-amplified with ErbB2, indicating that amplification of ErbB2 may impact its own hormonal regulation

Training Accomplishments

In the period of time since the last annual report I have become much more proficient in the technique of microinjection to create transgenic mice and have created several strains of mice. In addition to practicing the technique, I have now successfully taught two new students in the lab how to create transgenic mice. The ability to teach the technique of microinjection will serve as an important asset in the future, in addition to solidifying my understanding of all aspects of the technique. In addition to microinjection, I have gained experience in the creation of cell lines derived from primary tumors. Indeed, I have now identified three cell lines that overexpress ErbB2 at high levels. Further, I have gained experience in the purification of epithelial cells and their culture on Matrigel. This technique is an important asset when examining alveolar structures, as we are able to observe the results of stimulation with various substances. Indeed, this technique will reveal the ability, or inability, of mammary epithelium to form a ductal network, form alveoli and lactate in response to the appropriate stimuli. In collaboration with Frank Graham's laboratory, I have also now acquired the training and skills required to purify and infect cells with an adenovirus containing Cre recombinase to mediate excision of the loxP flanked constructs.

Academically, I have now completed all course work required for my Ph.D. degree and will begin my comprehensive examinations within several weeks. Upon completion of the comprehensive examination, I will be free to focus completely on my dissertation work as outlined in the statement of work.

Key Research Accomplishments

- **Generation of a mouse model of mammary tumorigenesis with remarkable similarity to the human condition exhibiting amplification and overexpression of ErbB2 and similar karyotypic events.**
- **Demonstration of mammary epithelium escaping the confines of a normal mammary gland by both the conditional activation of ErbB2 and the mammary specific expression of a putative ErbB2 ligand.**
- **Generation of cell lines from mammary tumors with amplification and overexpression of ErbB2.**
- **Completed FVB backcrossing of loxP flanked ErbB2 MMTV-Cre mice in order to examine impact of loss of ErbB2 on mammary gland development.**
- **Demonstration that Cre mediated excision resulting in ErbB2 null cells is possible by interbreeding with transgenic mice.**

Reportable Outcomes

- **Manuscript submitted to Oncogene (Attached as appendix one)**
- **Attended "Signalling in Normal and Cancer Cells", March 2-6, 2001, where Dr. Muller presented a portion of my work and I presented a poster. See abstract in appendix two.**

- Isolation of three cell lines from tumors with amplification and overexpression of ErbB2.
- Generation of a mouse model in the FVB background that will allow the examination of mammary gland development in the absence of ErbB2.
- Awarded the “Lee Nelson Roth Award”, an annual award to a doctoral student working in the area of cancer research at McMaster University based on the work described in section one.

Conclusion

Funding was provided for this project in order to address the role of ErbB2 in mammary gland development and tumorigenesis. After two years of funding, the most important result is the development of a mouse model of mammary carcinogenesis that mimics the human condition in several facets. Expression of an activated ErbB2 allele under the control of the endogenous promoter specifically in the mammary gland resulted in mammary adenocarcinomas that had amplified and overexpressed ErbB2. Additionally, these tumors share karyotypic abnormalities with the human condition. However, in addition to the tumorigenesis, this work has provided us with insights into the role of ErbB2 in normal mammary gland development. Expression of the activated ErbB2 allele results in mammary epithelium that escapes from the confines of the fat pad. This work suggests that deleting ErbB2 in the mammary gland may impact the outgrowth of the ductal network. The attempt to create a mammary gland lacking ErbB2 has been unsuccessful as MMTV directed Cre expression was lost after interbreeding with mice containing loxP flanked ErbB2. However, I have now backcrossed the tissue specific knockout mice into the FVB background to avoid potential transgene methylation problems and have initiated the crosses to delete ErbB2 in the mammary gland. This will now allow us to assess the role of ErbB2 in mammary gland development.

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Appendix One

Centrosome abnormalities, recurring deletions of chromosome 4, and genomic amplification of *HER2/neu* define mouse mammary gland adenocarcinomas induced by mutant *HER2/neu*

Cristina Montagana, Eran Andrechek, Hesed Padilla-Nash, William Muller and Thomas Ried

**Centrosome abnormalities, recurring deletions of chromosome 4, and
genomic amplification of HER2/*neu* define mouse mammary gland
adenocarcinomas induced by mutant HER2/*neu***

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Abstract

The conditional expression of activated HER2/*neu* gene under its endogenous promoter in the mammary epithelium of the mouse results in accelerated lobular development and focal mammary tumors. Carcinogenesis, however, requires amplification and considerably increased expression levels of oncogenic *neu*. Deducing from the multiple genetic aberrations required for human breast cancer to develop, we hypothesized that in addition to the over-expression of an activated HER2/*neu*, secondary aberrations would occur. We have therefore conducted a genomic screen for chromosomal imbalances and translocations using comparative genomic hybridization and spectral karyotyping. The results reveal a moderate degree of chromosomal instability and micronuclei formation in short-term cultures established from primary tumors. Genomic instability appears to be linked to the amplification of functional centrosomes, a phenomenon that we frequently observed in other tumor types. Seventy percent of the tumors revealed genomic amplification of HER2/*neu*, often in the form of double minute chromosomes, which correlated with recurring loss of mouse chromosome 4D-E, a region that is orthologous to distal human chromosome 1p. It is likely that this region contains putative tumor suppressor genes whose inactivation is required for tumor formation in this model of human breast cancer.

Introduction

Modeling human cancer in the mouse has become an increasingly valuable tool for understanding the genetic events responsible for tumor initiation and progression. Murine models can be used to monitor the effects of tissue specific elimination of tumor suppressor gene function and of the over-expression of cellular oncogenes. Numerous models for human breast cancer have been described (Hennighausen, 2000), many of which attempt to recapitulate the genetics of human breast cancer by over-expressing oncogenes known to be involved in the human disease. For instance, the forced expression of *c-myc*, *HER2/neu*, and *cyclinD1*, respectively, under the control of the mammary gland specific MMTV-promoter results in mammary gland adenocarcinomas (Muller *et al.*, 1988; Stewart *et al.*, 1984; Wang *et al.*, 1994). Accordingly, the conditional, tissue specific depletion of *BRCA1* function results in tumor formation (Xu *et al.*, 1999a; Xu *et al.*, 1999b) and the impairment of *p53* and *RB1* function via expression of SV40 large T antigen under the control of the C3 promoter induces *K-ras* amplification and tumorigenesis (Liu *et al.*, 1998). As in human cancers, it is likely that tumorigenesis in the mouse is promoted, or at least accompanied by, the acquisition of non-random chromosomal aneuploidies. For instance, human breast cancers frequently exhibit extra copies of chromosome 20, gains of chromosome arms 1q, 8q, and 17q, and losses that map to 8p and 17p (Ried *et al.*, 1995; Tirkkonen *et al.*, 1998). It would be intriguing to query whether this particular distribution of genomic imbalances is maintained in murine models of breast cancer despite the considerable shuffling of chromosomes during the course of evolution. Such comparative cytogenetic analyses,

however, have been hampered by the intricacy of reliably karyotyping mouse chromosomes. We have therefore used spectral karyotyping (SKY) (Liyanage *et al.*, 1996; Schröck *et al.*, 1996b) and comparative genomic hybridization (CGH) (Kallioniemi *et al.*, 1992; Weaver *et al.*, 1999), two techniques that overcome these hurdles, to characterize chromosomal aberrations in a mouse model of human breast cancer induced by the expression of an activated mutated HER2/*neu* under its endogenous promoter (Andrechek *et al.*, 2000). Genomic amplifications of the oncogenes HER2/*neu* and *c-myc* and potential deletions of the tumor suppressor genes *p53* and BRCA1 were assessed using fluorescence in situ hybridization. As a potential basis for the observed chromosomal aneuploidy we investigated centrosome integrity in these mammary cells using immunohistochemical visualization of γ -tubulin. We consider that such studies provide important baselines for the molecular analysis of murine tumors and contribute to the validation of mouse models for specific human cancers.

Results

The over-expression of an activated HER2/*neu* oncogene regulated via its physiological promoter results in the development of mammary gland adenocarcinomas in transgenic mice (Andrechek *et al.*, 2000). As part of the family of epithelial growth factor receptors, the HER2/*neu* oncogene is amplified in about 25% of human breast cancers (Revillion *et al.*, 1998). The amplification and over-expression of HER2/*neu* in human breast cancers is accompanied by additional genetic and chromosomal abnormalities and variations in the nuclear DNA content (aneuploidy). As for virtually all human carcinomas, these aberration result in the acquisition of specific genomic imbalances (Ried *et al.*, 1999). In breast cancers, regions of recurring genomic imbalance map to chromosome 1q, 8q, 17q, and 20 (gains) and 8p and 17p (losses). In many instances, such recurring imbalances point to the mapping position of oncogenes and tumor suppressor genes, respectively. Despite the fact that karyotype information could contribute to the validation of tumor models and provide entry points for the molecular cloning of cancer associated genes, data on chromosomal aberrations in murine carcinoma models remain sketchy.

In this study, we have applied molecular cytogenetic techniques, including SKY, CGH, and FISH with gene specific BAC clones to establish a comparative map of chromosomal aberrations in a series of 12 primary tumors induced by the expression of a constitutively activated HER2/*neu*. Karyotype analysis was performed on short-term cultures derived from primary tumors using SKY. An example of a SKY analysis is shown in Figure 1 for tumor 22. The most frequent chromosomal aberration was a deletion or monosomy of chromosome 4 (7 of 12 tumors). Except for the deleted

chromosome 4, we could not detect recurring structural chromosomal aberrations, however, clonal numerical aberrations (chromosomal aneuploidies) were common. One tumor, #21, was normal both by SKY and CGH analysis. We cannot exclude that this is due to the preferential culture of fibroblasts or normal breast epithelial cells. With the exception of two tumors, in which we observed a diploid and tetraploid (#22) and triploid stem line (#19), the cells were predominantly diploid. Karyotypes for all of the tumors are summarized in Table 1 and can be retrieved from the SKY database at <http://www.ncbi.nlm.nih.gov/sky/skyweb.cgi>. As a means in establishing a quantitative measure for intra-tumor heterogeneity, we quantified the variation of chromosome numbers in individual cells. Gains or losses relative to the modal copy number of each chromosome in the tumor resulted in chromosomal instability indices ranging from 0.2 - 4. This is lower than that observed in aneuploid cell lines established from human carcinomas (M. Difilippantonio, personal communication).

In order to define the deleted region of chromosome 4 with higher resolution, we used FISH with genetically mapped BAC clones to determine the smallest deleted interval. The hybridization pattern (Figure 2) indicated that only one chromosome retained both markers (D4Mit149 and D4Mit254). The breakpoint is therefore proximal to 53.6cM on the genetic map, which corresponds to chromosome bands 4E1-2. This region of mouse chromosome 4, which was deleted in all tumors with chromosome 4 aberrations, is orthologous to human chromosome 1p32-1p36.

48% of the metaphases from 10 tumors analyzed showed chromosomes that had acquired loss of the heterochromatic regions close to the centromere on band A1 (Figure 1). The most common chromosomes involved in aberrations were chromosome 8 (5 of

12 tumors), chromosome 9 (7 of 12 tumors), and less frequently chromosome 14 (3 of 12 tumors), chromosome 7, 18 and X (1 of 12 tumors).

The analysis of human carcinomas using SKY and CGH has revealed that the majority of chromosomal aberrations result in genomic imbalances (Ghadimi *et al.*, 1999), and that recurring copy number changes of particular chromosomes or chromosomal regions are a defining parameter of distinct human cancers (Ried *et al.*, 1999). In order to establish comparative maps of genomic imbalances, the series of 12 murine mammary gland adenocarcinomas was analyzed by CGH. The results are summarized in Figure 3 (see also the website <http://www.ncbi.nlm.nih.gov/sky/skyweb.cgi> for the CGH and SKY results). CGH confirmed the frequent loss of distal chromosome 4 as identified by SKY. High-level copy number increases that mapped to distal chromosome 11 were observed in 6 of 12 tumors, and low copy number increases in the same region were found in two additional tumors. In two cases (#20, #22) extra copies of chromosome 15 were detected. Both the *c-myc* oncogene and telomerase reverse transcriptase gene (hTERT) map to this chromosome. Less frequent copy number changes were observed on chromosome 2 (gained in #20/2 and #15) and 19 (gained in #5 and #22) and losses of the distal portion of chromosome 9 (#15, #18, #20). In total, we observed 34 copy number changes: divided by the number of cases, this translates to an average number of copy alterations (ANCA) of 2.8.

Eight of 12 tumors revealed metaphases containing extra-chromosomal DNA fragments whose shape was consistent with double minute chromosomes (DMs). The number of DMs ranged from 50 to 100 per cell (Figure 4 B-C). DMs in solid tumors are

frequently the cytogenetic correlate of oncogene amplification (Alitalo *et al.*, 1985). Since the amplicon identified by CGH mapped to the distal end of chromosome 11, we hypothesized that the *HER2/neu* gene, which maps to mouse chromosome 11 at 57cM was the target for this gene amplification. FISH with a BAC clone specific for *HER2/neu* confirmed our hypothesis. Seven tumors contained greatly increased copy numbers of this oncogene located on the DMs (Figure 4 B-C, Table 2). The genomic amplification detected by CGH is consistent with previous results using Southern-blot analysis with the exception of case #20, where CGH and FISH revealed greatly increased copy numbers for *HER2/neu*, however, amplification was not detected by Southern-blot analysis.

Genomic deletions frequently contribute to allelic loss and tumor suppressor gene inactivation in human cancers. We therefore investigated whether loss of the tumor suppressor genes *p53* and *BRCA1*, both involved in human breast cancers, were required for tumorigenesis in this mouse model. FISH analysis with a BAC clone for *BRCA1* clearly demonstrated that this tumor suppressor gene, which also maps to the distal end of chromosome 11 (Schröck *et al.*, 1996a), was excluded from the amplicon and was present in two copies in diploid cells. We could also show that inactivation of *p53* (at least via chromosomal deletion) was not necessary for tumorigenesis because two copies were consistently observed in the diploid cells. The *c-myc* oncogene was not present in copy numbers higher than the ploidy of mouse chromosome 15. Examples of the FISH experiments are provided in Figure 4 and the results compiled in Table 2.

With one exception, all tumors revealed predominantly numerical chromosomal aberrations (Table 1). Such aberrations can be due to compromised chromosome segregation fidelity during mitotic cell division. The centrosome organizes the spindle

apparatus prior to cell division and nucleates the proper assembly of microtubules whose attachment to the kinetochore at each chromosomes is essential for correct segregation (Kellogg *et al.*, 1994). We have previously shown that centrosome abnormalities occur exclusively in human colorectal carcinomas that are aneuploid, but not in their diploid, mismatch repair deficient counterparts (Ghadimi *et al.*, 2000). We therefore wanted to investigate whether abnormalities and amplification of functional centrosomes could, at least partially, contribute to the observed chromosomal aneuploidy. The centrosome can be visualized using an antibody against γ -tubulin, a protein that is concentrated in the centrosome during all stages of the cell cycle. The number of observed centrosomes in normal cells is cell cycle dependent as the centrosome is duplicated during S-phase. Five tumor samples were analyzed (#15, #18, #19, #20 and #23) and four of them revealed a significantly increased number of centrosomes. The average number per cell varied between 2.72 and 3.2, with up to 66% of the cells showing abnormal centrosome numbers. Besides the normal distribution of 1-2 centrosomes per cell (Figure 5A) we observed different patterns of centrosome abnormalities. In many instances, the centrosomes lined up similar to pearls on a string at the edge of the nucleus (Figure 5B). A second pattern displayed centrosomes located in the periphery of the cells and obviously detached from the nucleus (Figure 5C). The third pattern indicated centrosome replication in the absence of centrosome separation because clusters of centrosomes were observed near the nucleus (Figure 5D). Evaluation of the nuclear DNA counterstain also showed the frequent occurrence of micronuclei. Micronuclei are indicative of chromosome breakage, increased formation of dicentric chromosomes, and lagging chromosomes due to errors in cell cycle timing (Müller *et al.*, 1996). 25% of the cells

2
were accompanied by micronuclei (as determined by propidium iodide staining), and approximately 60% revealed aberrant centrosome numbers. Micronuclei occurred only rarely in cells with normal centrosome numbers (7%) but were frequently found in cells with additional centrosomes (18%). However, we observed a significant number of cells with centrosome abnormalities in the absence of micronucleus formation (45% of all cells analyzed).

Discussion

The acquisition of tumor specific patterns of chromosomal imbalances is a defining characteristic of cancers of epithelial origin. For instance, colorectal carcinomas almost invariably reveal copy number increases of chromosomes 7 and 13, and of chromosome arms 8q and 20q, while chromosome 18 is frequently lost (Ried *et al.*, 1996). Also, the emergence of these chromosomal aneuploidies occurs in a more or less defined sequence of events. Trisomy of chromosome 7 is the earliest events that can already be observed in small polyps (Heim & Mitelman, 1995). Such a linear progression is less well established during breast carcinogenesis, however, it is clear that mammary tumors display a non-random involvement of certain chromosomes. Cytogenetic and molecular cytogenetic analyses have clearly shown that a gain of chromosome arm 1q, as well as gains of chromosome arms 8q and 17q accompanied by losses of the respective short arms, are recurrently found in breast cancers (Heim & Mitelman, 1995). It is even possible to assign certain cytogenetic features to subgroups of breast cancers, that differ with respect to the nuclear DNA content, tumor size, lymph node status, and clinical course (Isola *et al.*, 1995; Ried *et al.*, 1995; Tirkkonen *et al.*, 1998). Therefore, it appears that the acquisition and maintenance of a distinct pattern of genomic imbalances is a feature that is strongly selected for and maintained despite intra-tumor heterogeneity and chromosome instability. With this information in mind it is intriguing to determine (i) whether mouse models of different carcinomas share this characteristic pattern of chromosomal aneuploidy, and (ii) whether this pattern is dependent upon the mode of tumor induction. We have previously reported the molecular cytogenetic evaluation of

mouse mammary gland adenocarcinomas induced by over-expression of the *c-myc* oncogene under the control of the MMTV-promoter (Weaver *et al.*, 1999). Here we extend such comparative molecular cancer cytogenetics to the evaluation of tumors induced by the conditional expression of an activated *HER2/neu* gene under the transcriptional control of its endogenous promoter (Andrechek *et al.*, 2000). During the course of mammalian chromosome evolution, human and mouse chromosomes have experienced a significant degree of reorganization (O'Brien *et al.*, 1999). Comparison of chromosome aberration maps could therefore, with increased resolution, pinpoint genes whose involvement in tumorigenesis is required across species boundaries. Such a comparison would be particularly interesting for chromosomes that are found in a specific aberration pattern in human cancers, for instance as an isochromosome. Human chromosome 17 serves as such an example: sequences on chromosome arm 17p are frequently lost (with *p53* as the target), while chromosome 17q is often gained (including *HER2/neu* and a yet to be identified second oncogene on human chromosome 17q23), occasionally via isochromosome 17q formation. Since *HER2/neu* and *p53* both reside on the same arm of mouse chromosome 11 chromosome translocations or interstitial deletions would have to occur if deletions of *p53* and maintenance of *HER2/neu* are both required for tumorigenesis. Our molecular cytogenetic evaluation has shown that the tumors in this model system do not require the acquisition of the equivalent of an isochromosome 17q in human breast cancers. While the genomic amplification of *HER2/neu* is required for tumorigenesis, as illustrated by the presence of DMs, deletions of *p53* are not present.

CGH analysis clearly confirmed the genomic amplification of mouse chromosome 11C-D, the mapping position of the oncogene *HER2/neu* (11D). Of note, the same percentage of tumors revealed loss of distal mouse chromosome 4. The acquisition of *HER2/neu* amplification appeared to be linked to chromosome 4 deletions; only one tumor showed a gain of 11D that was not accompanied by the loss of parts or the entire homologue of chromosome 4. We had previously observed deletions of mouse chromosome 4 in MMTV-*c-myc* induced mammary gland adenocarcinomas (Weaver *et al.*, 1999) and in conditional knockouts for the tumor suppressor gene *BRCA1* (Weaver *et al.*, submitted). These data therefore strongly suggest that a yet unidentified tumor suppressor gene resides at this chromosomal locus. This result is even more significant because distal mouse chromosome 4 is orthologous to human chromosome 1p. Human chromosome 1p has been shown in several reports to be subject to copy number loss in human breast cancers (Bieche *et al.*, 1994). One potential candidate gene is p73, a member of the p53 gene family (Chen *et al.*, 2001). Interestingly, in human neuroblastomas, genomic loss of chromosome 1p is associated with amplification of the *n-myc* oncogene, frequently in the form of DMs (Bieche *et al.*, 1994). One could hypothesize that the common and coordinate loss of chromosome band 4D and the emergence of DMs indicates that the function of a tumor suppressor gene in this region might extend to the suppression of gene amplification. The homology of distal mouse chromosome 4 also comprises region 9p21 in humans, which is the site of the p16INK4A and p19INK4d genes. These genes are involved in cellular senescence and immortalization and are therefore additional potential tumor suppressor candidate genes (Carnero *et al.*, 2000).

Our data also suggest that neither copy number increases of the *c-myc* oncogene, nor deletions of the tumor suppressor genes *p53* or BRCA1, were required for tumorigenesis in this model. We can, however, not exclude the possibility of functional inactivation of either of these tumor suppressor genes by mechanisms different from chromosomal deletions. This is in contrast to tumors in conditional knockout mutants for BRCA1, in which both cytogenetic and Western-blot analyses suggest the frequent inactivation of *p53* (Weaver et al., submitted).

CGH analyses of human carcinomas have provided evidence that tumor staging and tumor aggressiveness correlate with the degree of genomic instability as measured by the average number of chromosomal aberrations (ANCA). For instance, diploid breast carcinomas display an ANCA index of 2.4-5, whereas aneuploid carcinomas, whose prognosis is much worse, show an ANCA of 6.8-12 (Ried *et al.*, 1999). The ANCA index in the mouse models of human breast cancer that we have studied so far is in general similar to that observed in human carcinomas. In mammary gland adenocarcinomas induced by *c-myc* over-expression, the ANCA is 5.75, and in BRCA1-deficient aneuploid tumors 8.0. In the HER2/*neu* model only relatively few chromosomal aberrations were detected (ANCA=2.8). It also appears that those tumor models that were induced by a strong oncogenic stimulus show fewer copy alterations than those induced by the tissue specific deletion of tumor suppressor genes. The lower ANCA value occurs even in the presence of considerable genetic heterogeneity from one tumor cell to another. It seems that a strong oncogenic stimulus overcomes the need for the acquisition and maintenance of numerous recurring chromosomal imbalances. We conclude that the sequential inactivation of multiple tumor suppressor genes, and the gain

of function of several oncogenes by way of chromosomal abnormalities as seen in human breast carcinomas are reduced to the amplification of HER2/*neu* and loss of mouse chromosome 4D. One could extend this observation to the hypothesis that mouse tumor models induced by tumor suppressor gene deletion more closely reflect the nature of multi-step carcinogenesis that we observe in human epithelial cancers. Induction of tumors by oncogene over-expression, however, provides an excellent tool for analyzing the particular pathways in which these genes are involved.

Materials and Methods

Tissue culture and metaphase chromosome preparation:

Mammary epithelial cell lines were derived from primary tumors of 12 different MMTV-Cre Flox Neo NeuNT knock-in mice (average latency 400 days). Tumor bearing animals were sacrificed and cell lines prepared from tumor tissue as previously described (Amundadottir *et al.*, 1995; McCormack *et al.*, 1998). Metaphase chromosomes were prepared following exposure to colcemid arrest (3-4 hours, final concentration 100 µg/ml) and standard hypotonic treatment and fixation in methanol/acetic acid.

Molecular cytogenetic analyses:

Spectral karyotyping was performed as described (Liyanage *et al.*, 1996). Briefly, flow sorted normal mouse chromosomes were labeled with specific fluorochromes or fluorochrome combinations. After in situ hybridization images were acquired using an epifluorescence microscope (DMRXA, Leica, Wetzlar, Germany) connected to an imaging interferometer (SD200, Applied Spectral Imaging, Migdal HaEmek, Israel). Chromosomes were unambiguously identified using a spectral classification algorithm that results in the assignment of a separate classification color to all pixels with identical spectra (Garini *et al.*, 1996). Chromosome aberrations were defined using the nomenclature rules from the International Committee on Standardized Genetic Nomenclature for Mice (Davisson, 1994). Six to ten metaphases were analyzed for each tumor. For CGH, DNA was prepared using high salt extraction and phenol purification and labeled by nick-translation using biotin-11-dUTP (Boehringer Mannheim,

Indianapolis, IN). Genomic DNA from strain-matched mice was prepared and labeled with digoxigenin-12-dUTP (Boehringer Mannheim). Hybridization was performed on karyotypically normal metaphase chromosomes (FVB strain) using an excess of mouse Cot1-DNA (Gibco-BRL, Gaithersburg, MD). The biotin-labeled sequences were visualized with avidin-FITC (Vector Laboratories, Burlingame, CA) and the digoxigenin labeled sequences were detected with a mouse derived antibody against digoxigenin followed by a secondary rhodamine conjugated anti mouse antibody (Sigma-Aldrich, Milwaukee, WI). Quantitative fluorescence imaging and CGH analysis was performed using Leica Q-CGH software (Leica Imaging Systems, Cambridge, UK). BAC clones (Research Genetics, Huntsville, AL) containing locus specific sequences for the oncogenes *HER2/neu* and *c-myc* and the tumor suppressor genes *BRCA1* and *p53* and two BAC clones containing the markers D4Mit149 and D4Mit254 (RCPI23 mouse library, clones 215G3 and 201N14) were used in this study. FISH probes were labeled with biotin or digoxigenin by nick translation and hybridized to chromosome preparations derived from the primary tumors. After over night hybridization at 37°C, the slides were detected with FITC-conjugated avidin (Vector Laboratories, Burlingame, CA) and TRITC-conjugated anti-digoxigenin antibodies, respectively (Sigma-Aldrich, St. Louis, MO).

Immunohistochemistry:

Cells were cultured on Falcon chamber slides (Thomas Scientific, Swedesboro, NJ), washed in PBS, and fixed with MetOH (0°C) for 10 min and washed again in PBS. Incubation with a monoclonal anti γ -tubulin antibody (Sigma, St. Louis, MO) diluted

1:1000 in 3% goat serum/PBS was performed overnight at 37°C. The antibody complexes were detected with TRITC-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) and counterstained with DAPI. Gray level images were acquired using a CCD camera (CH250, Photometrics, Tucson, AZ) mounted on a Leica DMRBE epifluorescence microscope, and pseudo-colored using Leica Q-Fish software. Further details for all Materials and Methods can be found at <http://www.riedlab.nci.nih.gov/>

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Table 1:

Summary of aberrations identified by SKY and CGH analysis for 12 HER2/*neu* primary tumors.

tumor	SKY	CGH
5	40,XX, Del(9?A1),+19,+50~100dmin	-4,+11,Dp(11D)
6	40 XX	+6
15	38-40,XX,+2, Del(4D-E), Del(8?A1)+50~100dmin	-13,Del(9F),Dp(11D),Del(14E)
15-17	37-54, XX, +X, Del(7?A1), -12,Del(14?A1), Del(18?A1), 50~100dmin	nd
16	38-40,XX,Del(4D-E), +6+50~100dmin Del(8?A1)	Dp(11D)
18	40-41,XX, Del(8?A1), Del(9?A1), Del(14?A1)	Del(9F),Dp(11D)
19	39-59,XX,+X, Del(8?A1), Del(9?A1)+11,-19,+50~100dmin	Del(4E),Dp(11D),Del(14E)
20	40-41,XX, Del(9?A1)+15,+50~100dmin	Del(9F),Dp(11E)
20/2	40-41, XX, +2,Del (4D-E),Del(9?A1),Del(14?A1)	-11
21	40 XX	
22	40-87,XX, Del(4C-E),+5, Del(8?A1),Del(9?A1)+15,+19, 50~100dmin	Dp(11D)
23	37-41XX,+X,-4, Del(9?A1)+10,+50~100dmin	Dp(11D)

Column 2 summarizes the karyotypes deduced by SKY. Column 3 shows the additional chromosomal gains and losses for each tumor as detected only by CGH.

Table 2:

Summary of FISH experiments.

tumor	% cells +DM's	p53 deletion	BRCA1 deletion	c-myc amplif.	centrosomes number
5	100	nd	nd	nd	nd
6	nd	nd	nd	nd	nd
15	76	-	-	-	0-8
15-17	2	-	-	-	nd
16	96	-	-	-	nd
18	nd	nd	nd	nd	0-7
19	85	nd	nd	nd	1-2
20	50	-	-	-	0-10
20/2	nd	nd	nd	nd	nd
21	nd	nd	nd	nd	nd
22	81	-	-	-	nd
23	100	-	-	-	0-6

Column 2 indicates the percent of cells containing double minute chromosomes for each sample. Columns 3, 4 and 5 show the results of FISH experiments with the BAC clones for the *p53* and BRCA1 tumor suppressor genes and for the *c-myc* oncogene, respectively. Column 6 displays the enumeration of centrosome numbers using an antibody against γ -tubulin. Not determined, nd.

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Figure legends

Figure 1:

Spectral karyotyping (SKY) of mammary tumor cultures derived from MMTV-Cre Flox Neo NeuNT mice. A representative metaphase is given for #20/2. The karyotype is 43XX, +5, +15, +19, Del(4D-E), Del(7A1), Del(14A1). Arrows denote the Del7 and Del14.

Figure 2:

FISH experiment with two BAC clones specific for chromosome 4. A triploid metaphase derived from tumor 22 is displayed. The yellow signals reflect the hybridization of BAC clone 219G3, which maps at 53.6 cM. The green signals refer to clone 206N14, at the genetic locus 76.5 cM. The arrows in the box insert identify the signals in the normal alleles revealing two intact loci. The homologous chromosomes show no hybridization signals for either of the clones, suggesting a deletion of the region 53.6-76.5 cM.

Figure 3:

Summary of gains and losses in 12 mammary gland tumors based on CGH-analysis. The lines left to the ideogram denote genomic losses and the lines right to the chromosome ideogram gains of chromosomes. Bold lines indicate high-level copy number increases (amplifications). Note the frequent deletions on chromosome 4 and gains on chromosome 11. Individual case numbers are provided on top of the gain and loss bars.

Figure 4:

A: Dual color FISH analyses of tumor 22 using a painting probe for chromosome 11 (blue) and a BAC clone specific for the *HER2/neu* oncogene (yellow). This tetraploid cell reveals four copies of an intact chromosome 11. No DMs were visible.

B: In contrast to the metaphase shown in A, the cell shown here reveals multiple DMs that labeled positively with the FISH probe for *HER2/neu* (yellow signals). Arrows denote the normal chromosome 11.

C: The interphase nuclei of tumor 22 show the amplification domains of *HER2/neu* after hybridization with the same FISH probe used in A and B.

D: FISH with a BAC clone for the tumor suppressor gene *BRCA1* shows two signals on chromosome 11 in this diploid metaphase (tumor 22).

E: FISH with a BAC clone for the tumor suppressor gene *p53* did not reveal small interstitial deletions (yellow signals) in this tetraploid cell of tumor 22.

F: Tumor 22 reveals an extra copy of the *c-myc* oncogene on chromosome 15 (yellow). However, the copy number of the *c-myc* oncogene does not exceed the copy number of chromosome 15, hence no amplification.

Figure 5:

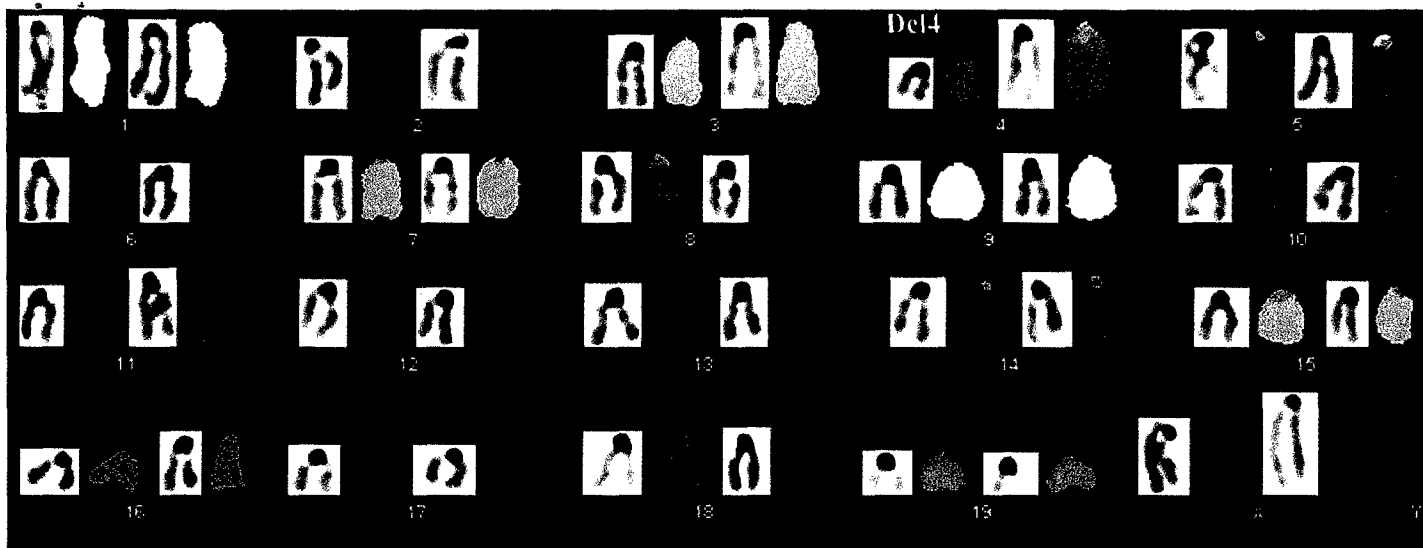
Immunocytochemistry with an antibody against γ -tubulin detects centrosomes. We observed four distinct patterns.

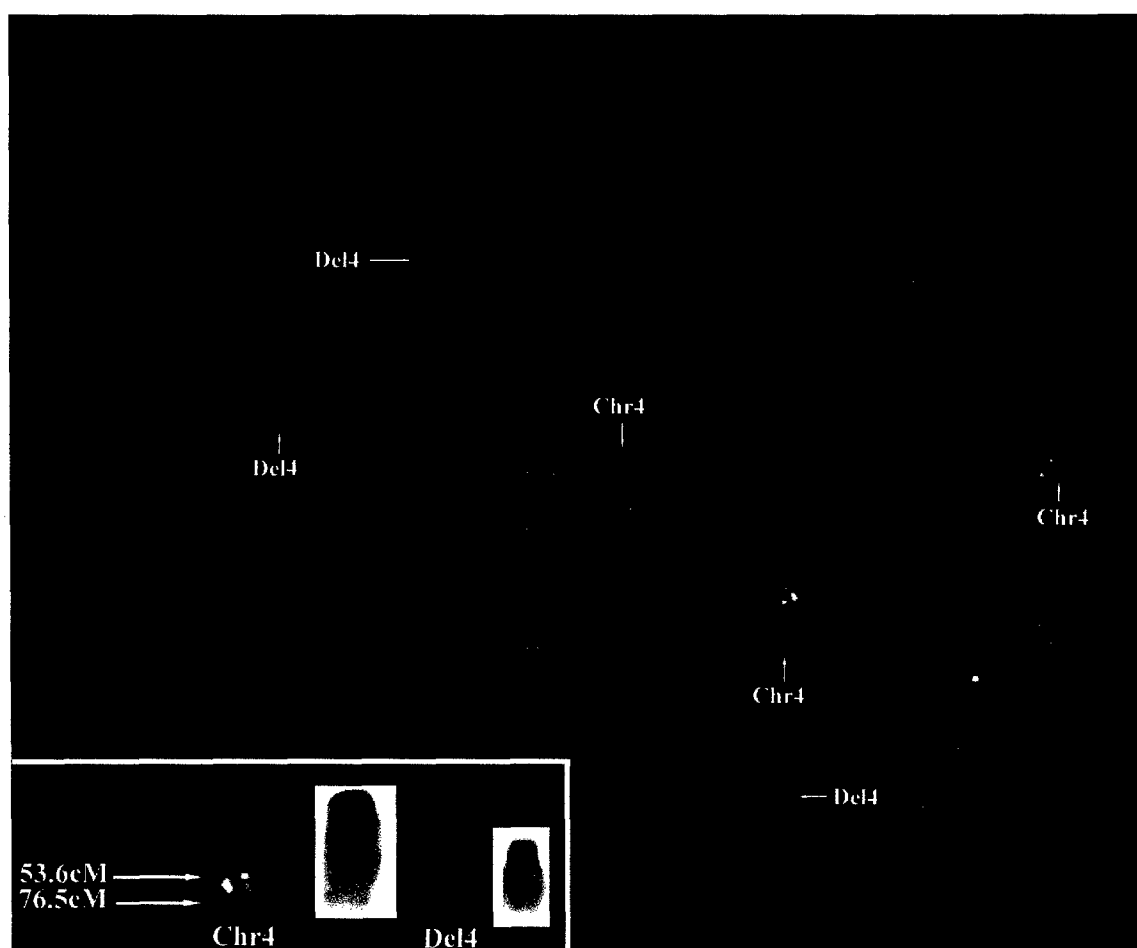
A: normal centrosomes in an interphase nucleus.

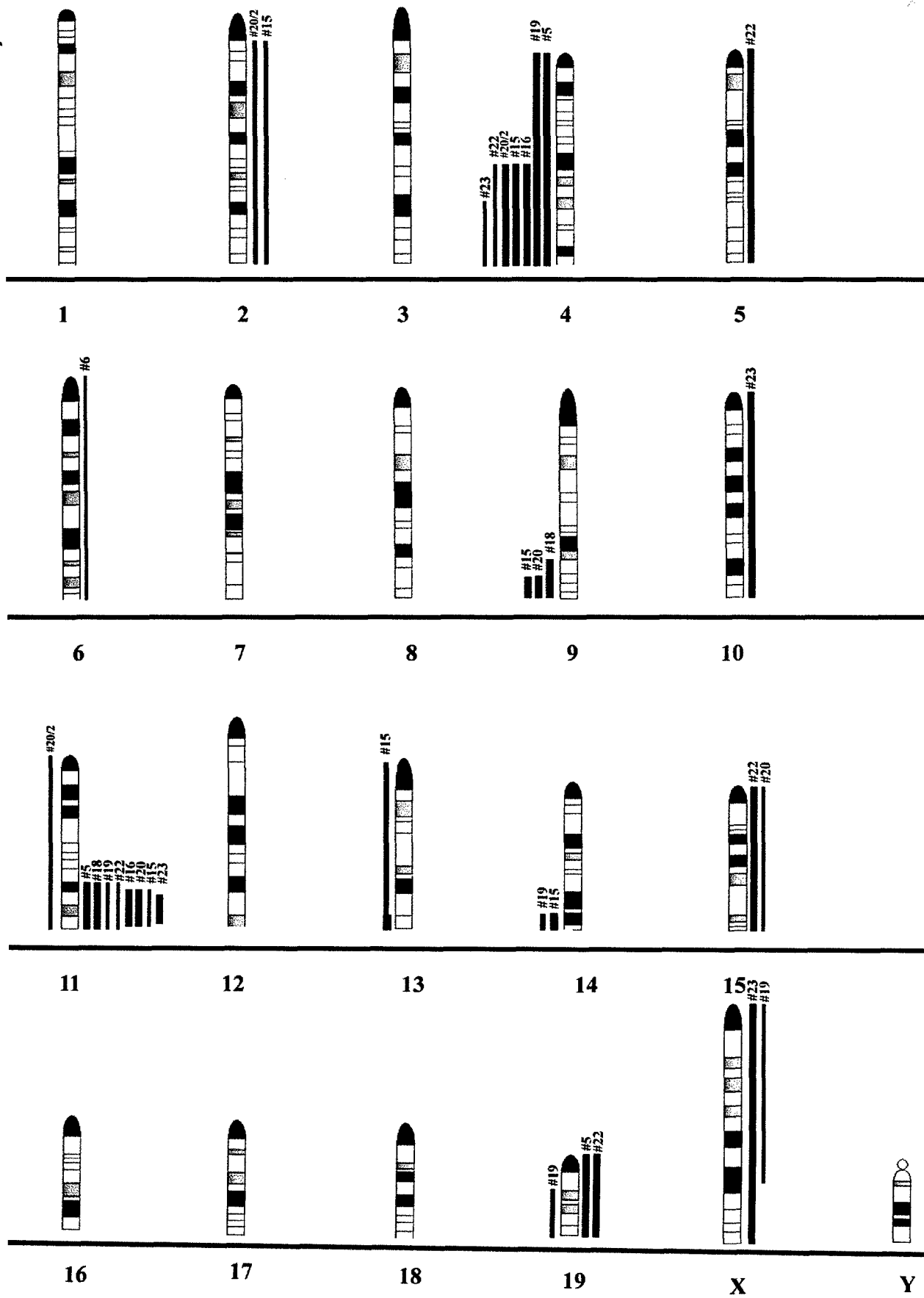
B: Increased centrosome numbers that are aligned at the periphery of the nucleus. Note the presence of micronuclei.

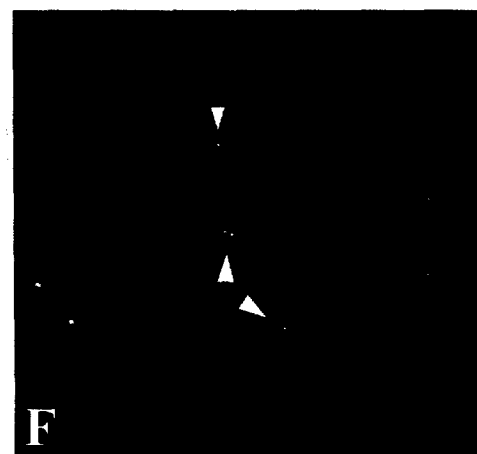
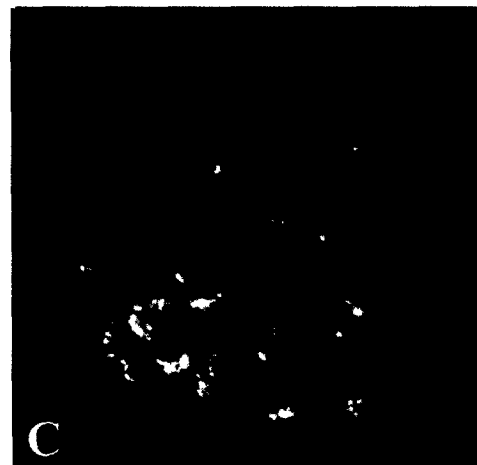
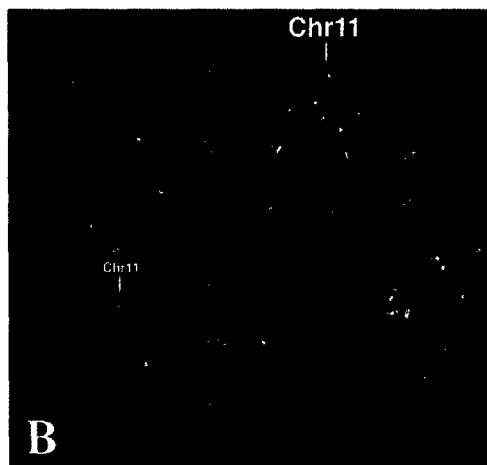
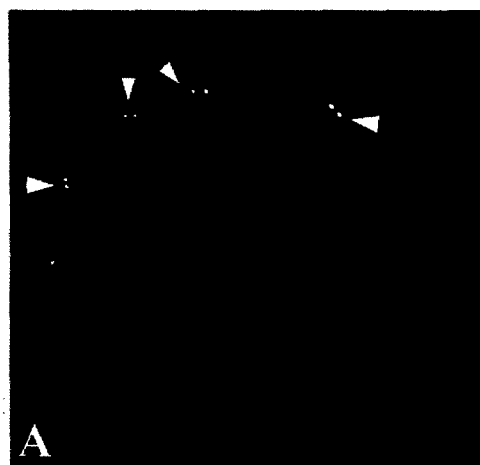
C: Detached centrosomes in an apparently apoptotic cell.

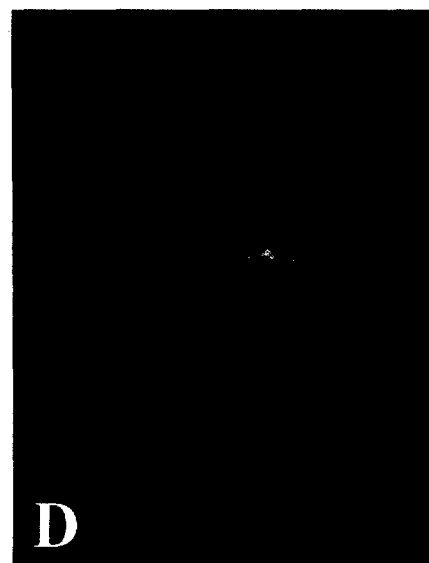
D: Multiplied, but non separated centrosome in an interphase nucleus.











Appendix Two

**Contains an abstract from;
"Signalling in Normal and Cancer Cells", March 2-6, 2001**

Genetic dissection of *erbB-2*/Neu signaling in tumorigenesis and development.

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Recent evidence has suggested that overexpression and amplification of *c-erbB-2* is an important determinant in the initiation and progression of a number of human cancers including breast, ovarian and lung. Although these studies suggest that *c-erbB-2* is a central player in these cancers, direct evidence supporting this contention is lacking. In addition to its documented role in pathological processes such as cancer, there is increasing body of evidence implicating *erbB-2* as an important gene in the development of muscle, neural and epithelial cell lineages. To test the importance of *erbB-2* in both development and cancer, we have generated specific loss of function (LOF) or gain of function (GOF) *c-erbB-2* alleles in the germline of transgenic mice.

Mammary specific expression of activated *erbB-2* under the transcriptional control of its endogenous promoter initially resulted in accelerated lobulo-alveolar development. However, focal mammary tumors arose in these strains after a long latency period suggesting that expression of activated *erbB-2* was not sufficient for mammary tumorigenesis. Strikingly, all mammary tumors derived from these strains bear amplified copies of the activated *erbB-2* allele relative to the wild-type allele and express highly elevated levels of *erbB-2* transcript and protein. Thus, like human *erbB-2*-positive breast tumors, mammary tumorigenesis in this mouse model requires the amplification and commensurate elevated expression of the *erbB-2* oncogene.

Appendix Three

Contains Figures One to Nine

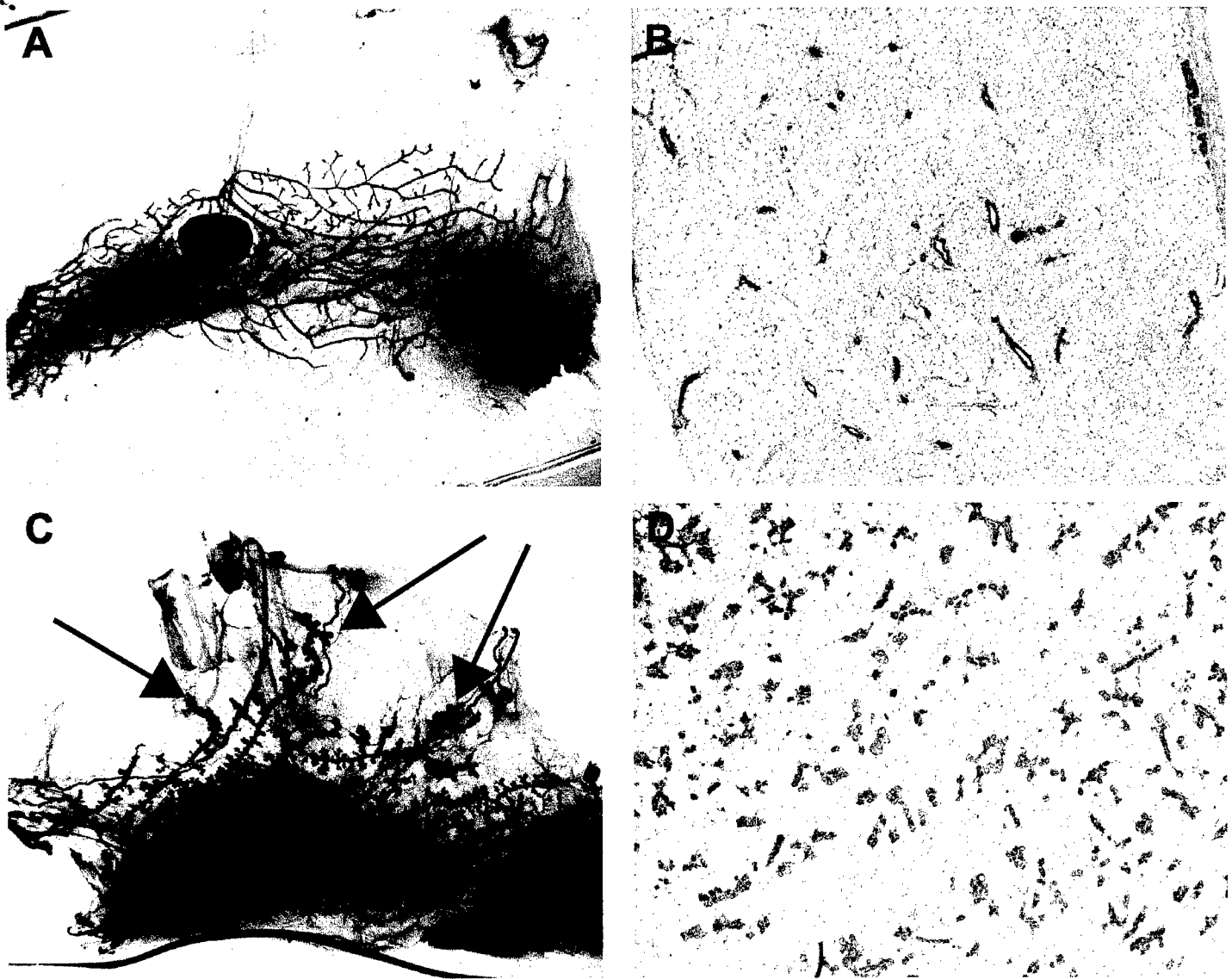


Figure One - Outgrowth of Mammary Epithelium

Wild Type (A and B) wholemounts and histology are shown. Note how the mammary epithelium is confined within the stroma in A. Expression of activated ErbB2 under control of the endogenous promoter (FlneoNeuNT MMTV-Cre mice) results in mammary epithelium that is able to escape the normal confines of the stroma (arrows in C). It should be noted that the mammary epithelium tends to escape the fat pad by invading the stroma surrounding the blood vessels. The mammary gland also exhibits the increased side branching previously described. The histology associated with the mammary gland is shown in D.

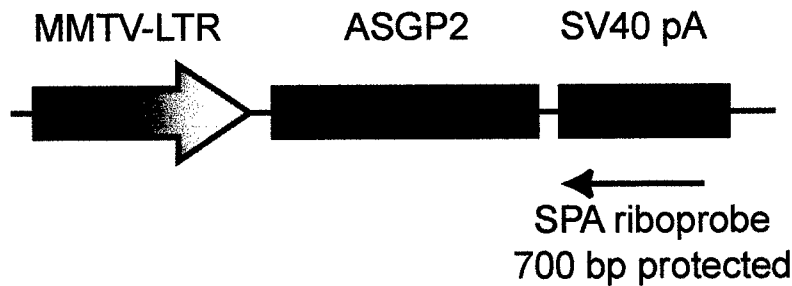
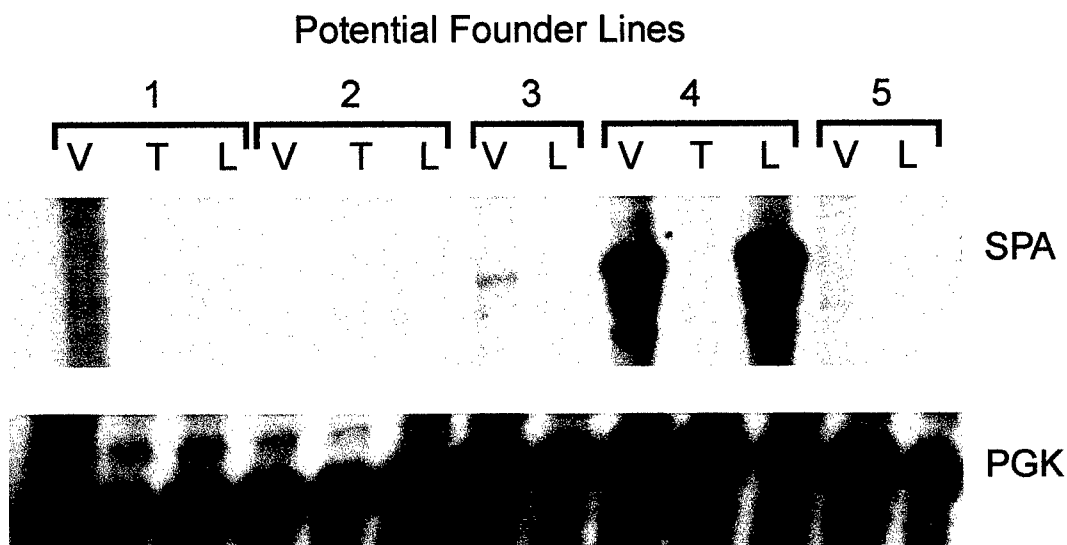
A**B**

Figure Two - Creation of Mice Expressing a Putative ErbB2 Ligand under Control of MMTV
 The construct used to create the transgenic mice is shown (A), where the Mouse Mammary Tumor Virus Long Terminal Repeat Promoter / Enhancer is used to drive expression of ASGP2, a putative ligand for ErbB2. RNA from Testes (T), virgin (V) and lactating mammary glands (L) was collected and screened using the SPA riboprobe illustrated (A). The RNase protection using this riboprobe is shown (B). PGK was included as an internal loading control. This demonstrates the creation of one line of transgenic mice expressing the transgene at high levels in the mammary gland (ASGP2-4).

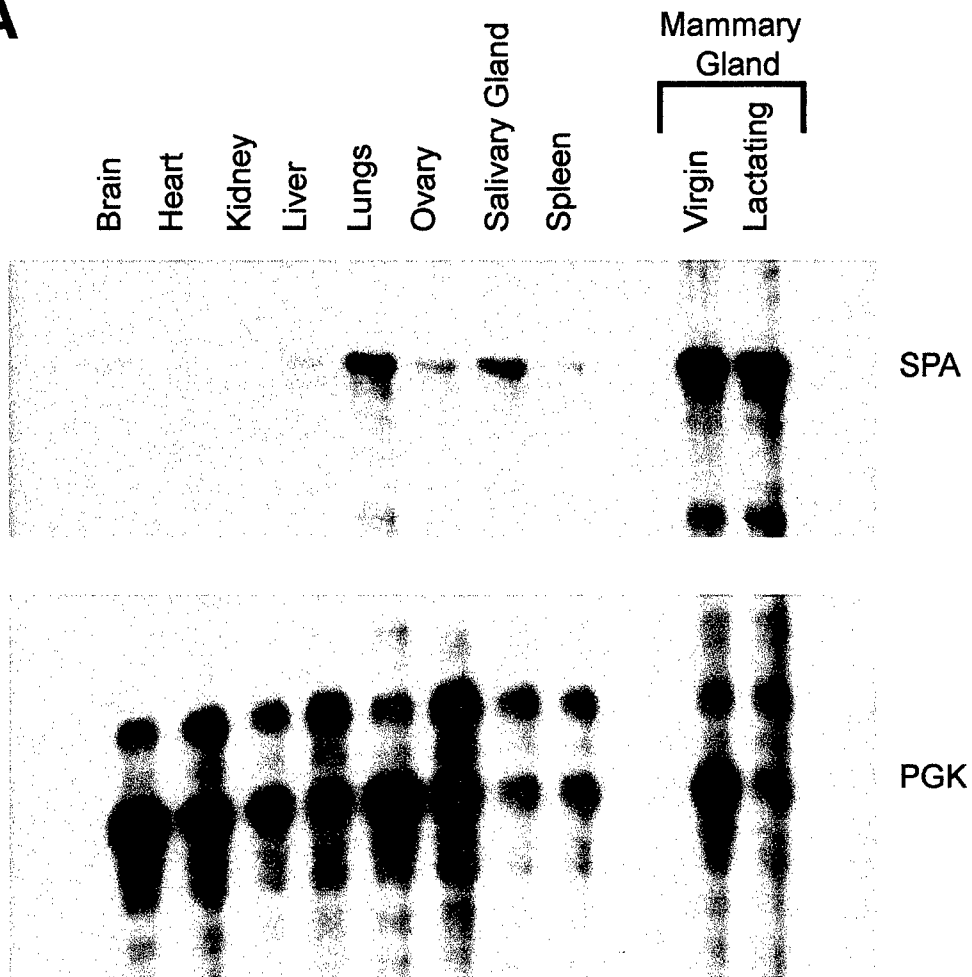
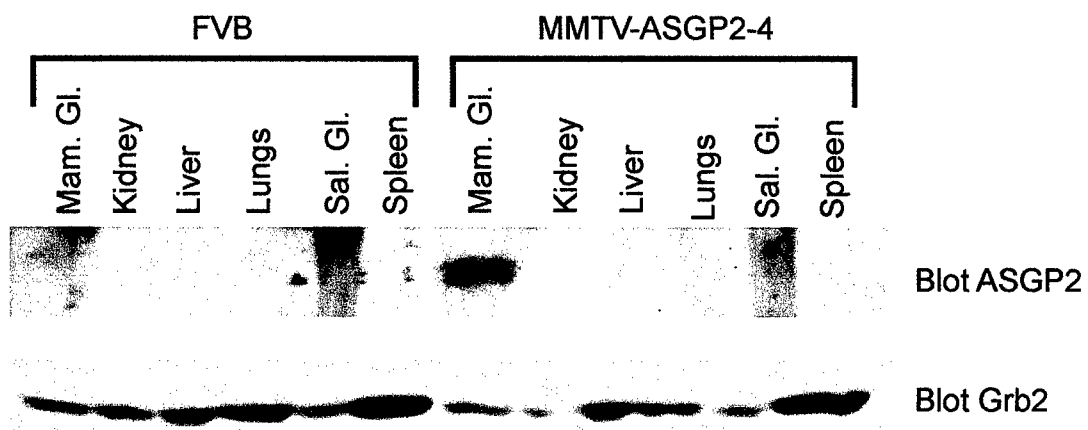
A**B**

Figure Three - Expression of the ASGP2 transgene is highly specific to the mammary gland. RNA was harvested from various tissues in the ASGP2-4 line of transgenic mice and was subjected to an RNase protection using the SPA riboprobe. The PGK riboprobe was included as an internal control for loading. This shows that expression is fairly tightly contained in the mammary gland (A). This was further illustrated by a western for ASGP2 where the protein was only detected in the transgenic mammary glands overexpressing ASGP2 (B).

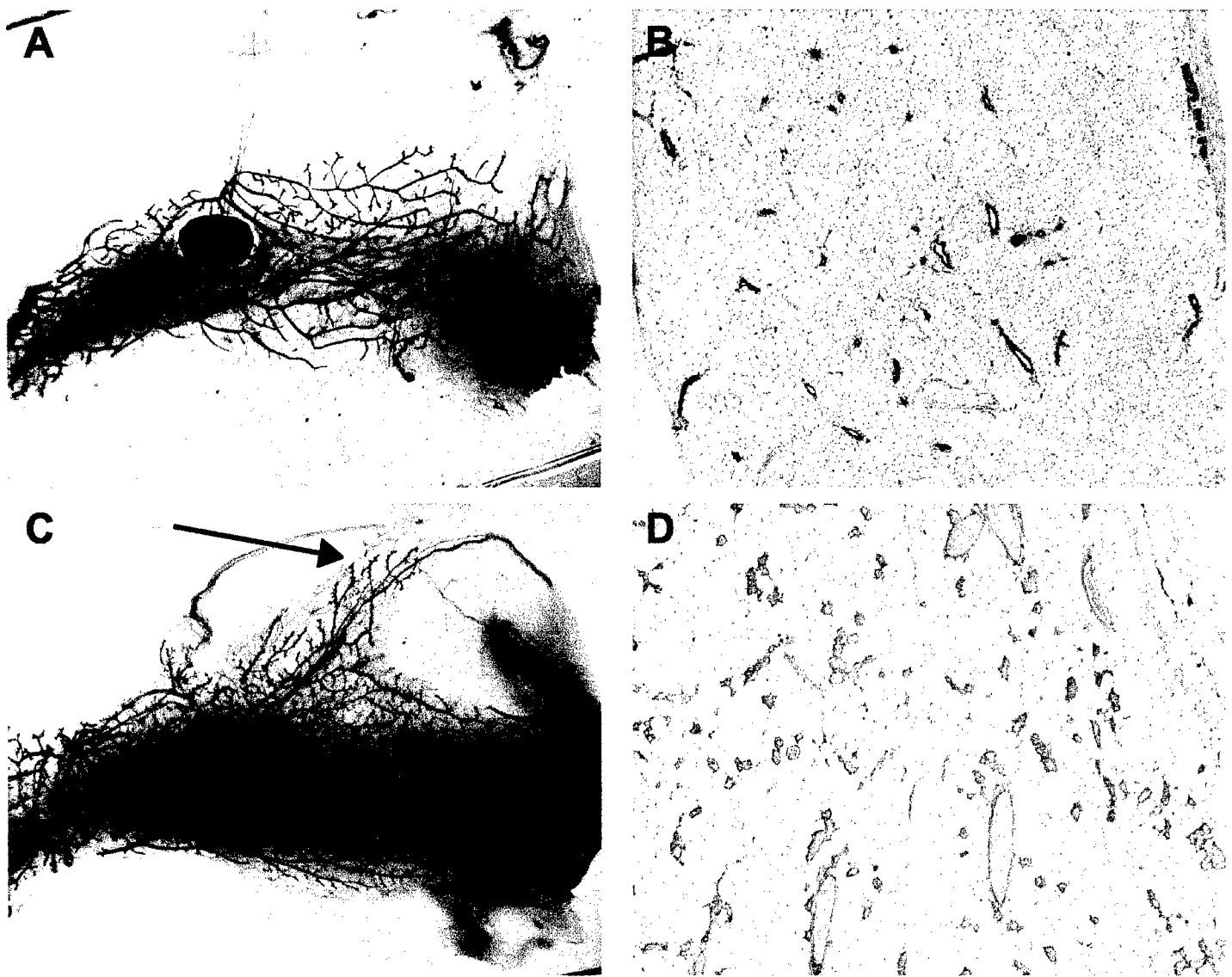


Figure Four - Outgrowth of Mammary Epithelium overexpressing a potential ErbB2 ligand. Wild type mammary gland wholemounts and histology are shown (A and B). A wholemount and histology from mice overexpressing a putative ligand for ErbB2, ASGP2, is also shown (C and D). Note the outgrowth from the mammary gland (arrow in C) which follows a blood vessel. The similarity of this phenotype to the phenotype illustrated in Figure One (C) is remarkable.

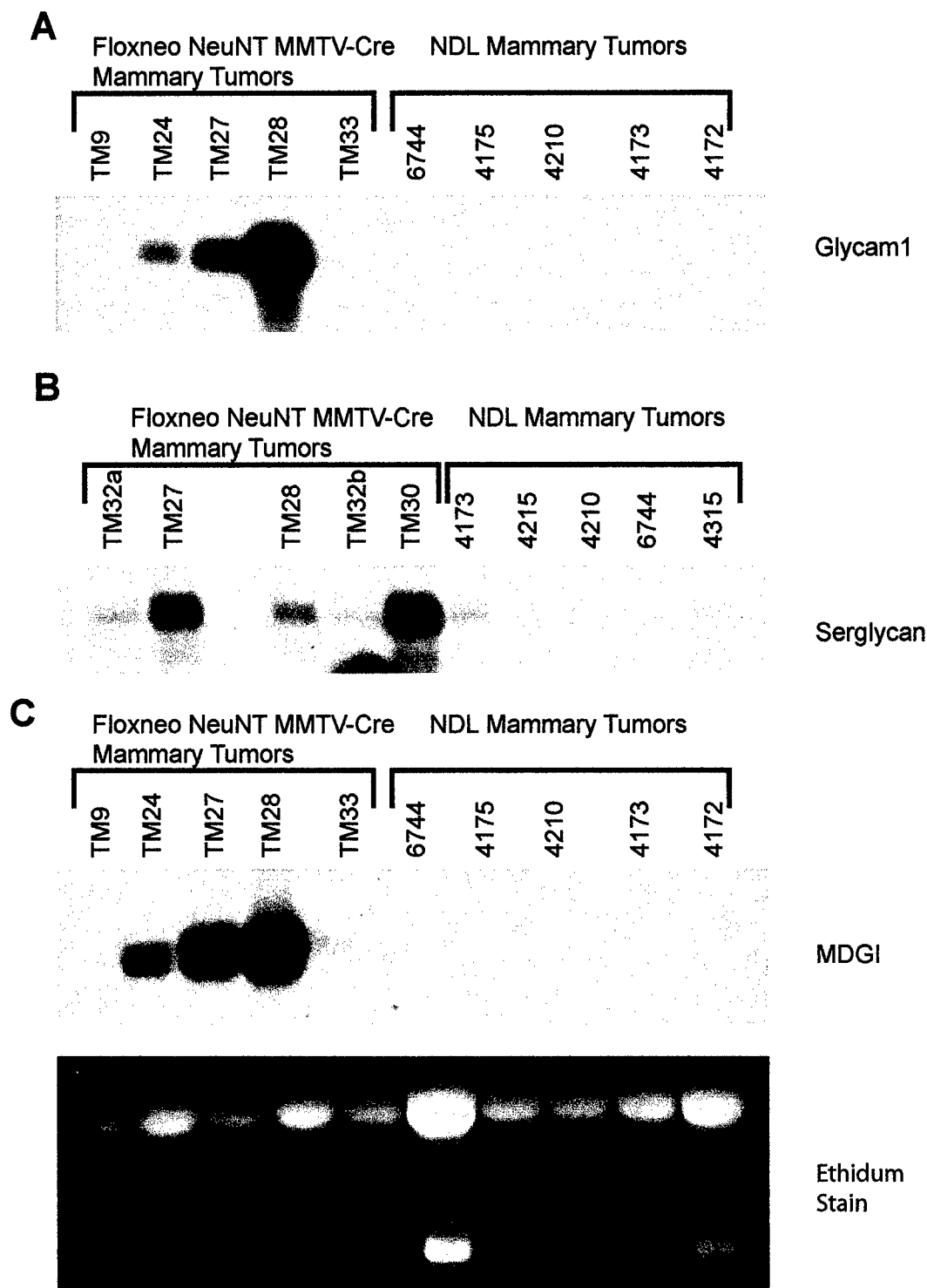


Figure Five - Confirmation of Chip Data by Northern Analysis

As an example of the Northern blots done as confirmation of the chip data (Table One), three Northern blots are shown. Northern blots for Glycam1 (A), Serglycan (B) and Mammary Derived Growth Inhibitor (MDGI) (C) are shown. Although data from the chip experiment indicated that Glycam1 was elevated 144 fold and MDGI was elevated 22 fold, we were unable to detect these genes in NDL samples in a Northern blot. Phosphorimager analysis of serglycan revealed a range of fold overexpression, but the average was an 8 fold overexpression, correlating well with the chip average of 10 fold. An ethidium stain was included to show loading error.

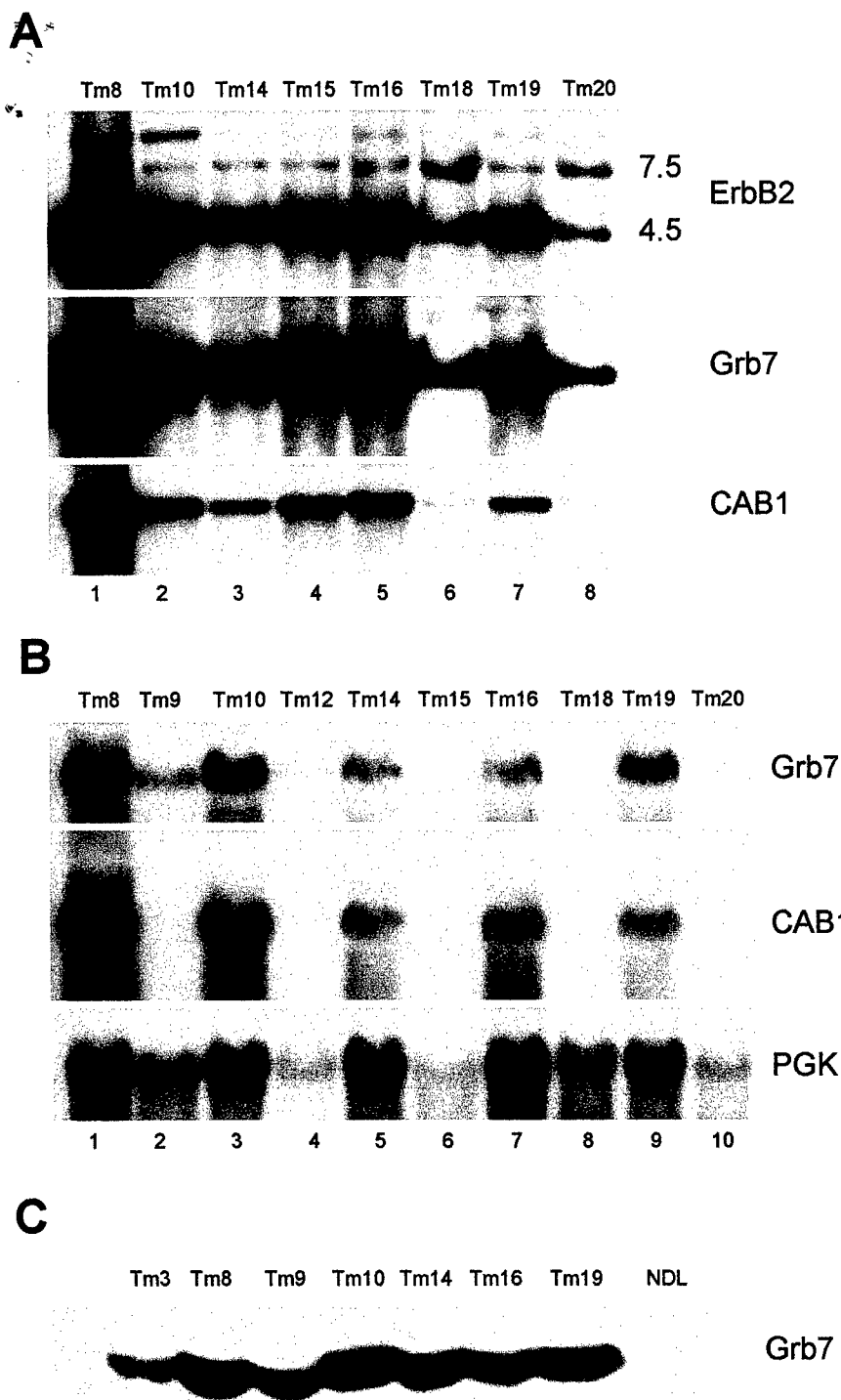


Figure Six - Amplification of Genes surrounding ErbB2

After examination of the data illustrated in Table One, several genes that appeared to be overexpressed in the conditionally activated tumors were examined due to their proximity to ErbB2. A southern blot (A) shows that for tumors with ErbB2 amplification (WT - 7.5kb, NeuNT - 4.5 kb), there is a corresponding amplification of both Grb7 and CAB1 (Note Tm18 and 20 are not amplified). Northern blots revealed that the tumors contained elevated levels of RNA transcripts for these genes which was not observed in the NDL tumor controls (data not shown). A single exposure is shown, at higher exposures the elevated levels are obvious for samples Tm9 and Tm12. Sample Tm15 did not contain high levels of RNA, as seen in the PGK controls (B). A Western blot for Grb7 also revealed high levels in tumors expressing high levels of RNA. Also included in this blot is a NDL sample showing the normal levels of Grb7 (C). These results suggest that genes surrounding ErbB2 are also amplified and should be considered when examining the phenotype.

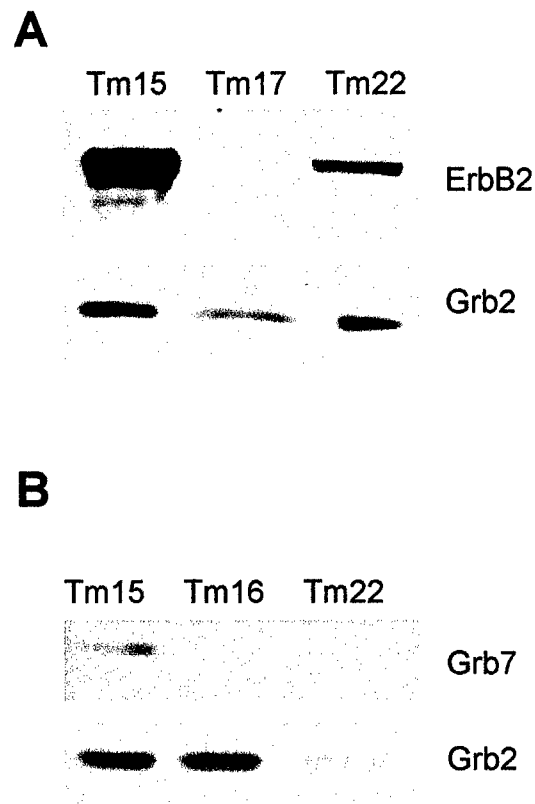


Figure Seven- Isolation of Tumor Cell Lines Overexpressing ErbB2 and Grb7

Screening of various cell lines generated from the tumor samples indicated above the lanes resulted in the identification of tumor cell lines overexpressing ErbB2. Initially, three cell lines were screened for ErbB2 expression resulting in the identification of two cell lines overexpressing ErbB2 (A). A third cell line was later identified that expressed ErbB2 at high levels (not shown). Of the three cell lines that overexpress ErbB2, only Tm15 was also shown to overexpress Grb7 (B).

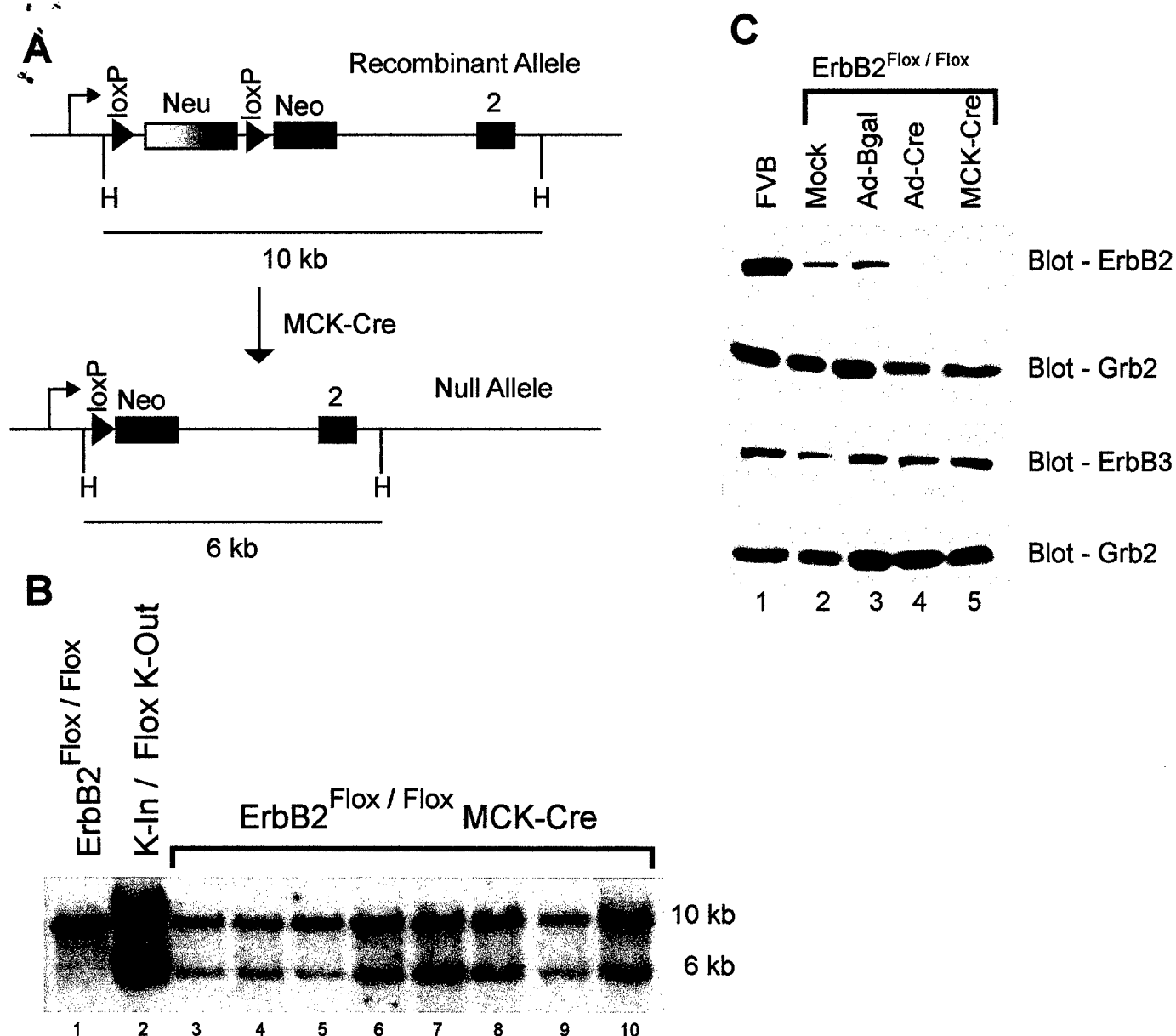


Figure Eight - The loxP flanked Neu allele is functional

Since interbreeding MMTV-Cre and the loxP flanked *ErbB2* mice did not result in excision of the loxP flanked cDNA, we tested the construct for transgene mediated excision. To accomplish this we used the MCK driven Cre Recombinase to achieve excision, creating a null *ErbB2* allele (A). In a Southern analysis, we examined excision in muscle using Neomycin as a probe in a Hind III digest. This clearly revealed that excision occurred in tissue expressing the MCK transgene (B). To determine whether the null allele also resulted in an absence of *ErbB2* protein, we examined muscle tissue in culture with adenoviruses expressing B-gal or Cre recombinase. Importantly, upon complete excision we observed a lack of *ErbB2* when blotting for *ErbB2* (compare lanes 1-3 vs 4-5 in C). Importantly, the *Grb2* loading control revealed that loading was approximately equal. Further, *ErbB3* levels were found to remain constant. These results illustrate that the loxP flanked cassette is functional, and given the previous demonstration of the utility of the MMTV-Cre transgenics the interbreeding should result in a mammary specific null allele for *ErbB2* in the FVB background.

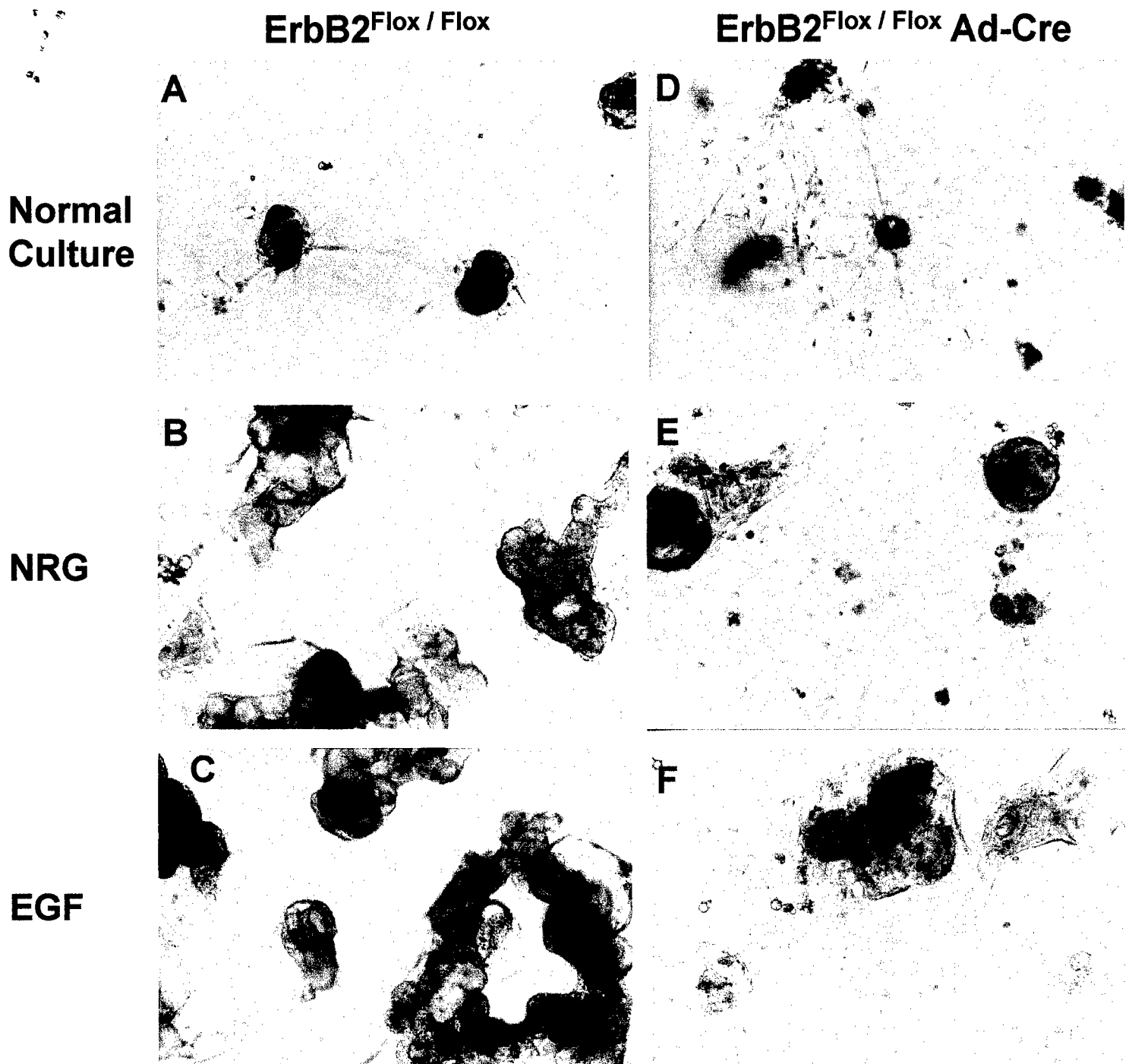


Figure Nine - Preliminary Differences in Mammosphere Culture due to Lack of ErbB2
Preliminary results in the culture of loxP flanked ErbB2 mammary epithelial cells without Cre Recombinase (A-C) and with an adenovirus expressing Cre Recombinase (D-F) are shown. While there are no large differences in normal culture conditions, the addition of growth factors results in minor differences. What is striking about these results is that the level of ErbB2 is only 10% of wild type levels in the loxP flanked ErbB2 mice and the mammospheres are still able to form normally. This may indicate that ErbB2 is dispensable for alveolar development and given the branching observed in the overexpression (Figure One), may be involved in regulation of ductal elongation and branching. This will be confirmed in a thorough examination of the MMTV-Cre Floxed ErbB2 mammary glands.

Appendix Four

Contains Table One

Gene	Accession Number	Fold Elevation	Confirm	Chromosomal Location
Differentiation Markers				
Glycam1	m93428	144.4	Northern	15 - 63.0 cM
WAP	j00649	128.6		11 - 0.5 cM
keritan-epidermal	m13806	99.1		Undefined
PSP	X01697	43.6	Northern	2 - 88.0 cM
von Ebner Salivary	U46068	32.8		2 - 76.3 cM
epsilon-casein	v00740	26.8		Undefined
keritan-epidermal	k02108	23.7		Undefined
cytokeritan	m28698	23.6		Undefined
MDGI	u02883	22.3	Northern	4 - 61.0 cM
alpha-amylase	v00719	20.9		Undefined
Proliferin	k02245	12.7	Northern	13 - 6.0 cM
Gamma-casein	d10215	8.4		5 - 45 cM
alpha-casein	m36780	5.4		5 - 44.9 cM
beta-casein	x04490	5.4		5 - 45.0 cM
Glycoproteins				
AEG-1	m92849	153.21		17 - 21.0 cM
Serglycan	x16133	10.6	Northern	Undefined
MUC1	m65132	5		3 - 44.8 cM
Clusterin	i08235	2.5		14 - 28.0 cM
MUC10	U37531	-53.6		Undefined
Transcription Factors				
HMG-1(y)	j04179	14.3	Western	17 - 13.2 cM
MSG1	msa.2467.0	9.4	Northern	Undefined
Krox-20	x06746	8.5		10 - 35.0 cM
PEA3	x63190	6.6		11 - 60.0 cM
ELF3	af016294	5.7	Northern	Undefined
Albumin D-box	u29762	-11.5	Western	7 - 23.0 cM
Amplicon				
Grb7	aa216863	38	Western	11 - 57.0 cM
CAB1	Msa.2277.0	26.3	Lightcycler	Undefined

Table One - Comparison of NDL and FlneoNeuNT MMTV-Cre mediated Tumorigenesis

After an RNA Chip experiment to compare RNA from MMTV-NDL and tumors from the conditional activated mice, the results were compiled and the major results are shown above. When completed, the method for confirmation of the chip data is stated. The fold elevation stated is the elevation in the conditionally activated samples as compared to the NDL samples. What is interesting to note is that numerous differentiation markers are elevated in the tumors where NeuNT was amplified. Interestingly, these tumors were less invasive and were focal in origin.



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218


SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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